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Developments in mycotoxin analysis: an update for 2010-2011

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Abstract

This review highlights developments in mycotoxin analysis and sampling over a period between mid-2010 and mid-2011. It covers the major mycotoxins: aflatoxins, *Alternaria* toxins, ergot alkaloids, fumonisins, ochratoxin, patulin, trichothecenes, and zearalenone. Analytical methods for mycotoxins continue to be developed and published. Despite much interest in immunochemical methods and in the rapid development of LC-MS methodology, more conventional methods, sometimes linked to novel clean-up protocols, have also been the subject of research publications over the above period. Occurrence of mycotoxins falls outside the main focus of this review; however, where relevant to analytical method development, this has been mentioned.

Keywords: aflatoxin, *Alternaria*, ergot, fumonisin, ochratoxin, patulin, trichothecene, zearalenone, sampling

1. Introduction

This review continues from a previous paper covering mycotoxin analytical developments over the period mid-2009 to mid-2010 (Shephard *et al.*, 2011). As in the previous paper, this review emphasises new methodology published over the titular period, although some novel natural occurrence data have been included at the discretion of the authors, especially where it demonstrates the applicability of new methods.

Fifty years have passed since the discovery of the aflatoxins and the commencement of modern research efforts on mycotoxins. However, the effects of mouldy food on human and animal health were known long before then. The prehistory of mycotoxins with particular reference to ergotism and alimentary toxic aleukia has recently been thoroughly reviewed (Ramos *et al.*, 2011). In contrast, Paterson and Lima (2010) have assessed the future effects of climate change on mycotoxins in food. Our current knowledge about human exposure and the health effects of deoxynivalenol (DON), nivalenol (NIV), T-2 and HT-2 toxins (T-2 and HT-2), zearalenone (ZEA), moniliformin, beauvericin (BEA) and the enniatins were the subject of a special issue of World Mycotoxin Journal (Volume 3, issue 4, 2010). Other recent reviews include aspects of the current state of analytical methods (Köppen *et al.*, 2010), the chemistry and biology of mycotoxins (Bräse *et al.*, 2009), patent activity in the mycotoxin analytical area (Anfossi *et al.*, 2010a) and developments in biological and synthetic binders for immunoassays of small molecules including mycotoxins (Fodey *et al.*, 2011). Woodhead Publishing

has published a book entitled 'Determining mycotoxins and mycotoxigenic fungi in food and feed', edited by S. De Saeger (ISBN 978-1-84569-674-0).

2. Sampling and sample preparation

Improving sampling and sample preparation methods used to detect mycotoxins and other quality attributes in food and feed products continues to be a high priority among regulatory agencies, international organisations, and commodity industries worldwide. Examples of several worldwide efforts are discussed below.

Brera *et al.* (2010) studied the variability and distribution of aflatoxin among ears of maize harvested from a field. The objective of the study was to measure the performance of sampling plan designs to estimate aflatoxin B_1 (AFB_1) contamination in field maize. The mean and variance among aflatoxin values for each ear were 10.6 µg/kg and 2,233.3 µg/kg, respectively. The large variance relative to the mean aflatoxin in the field indicates distribution among ear aflatoxin values is extremely skewed. From the variance and distribution measurements, operating characteristic curves were developed to show the performance of various sampling plan designs to estimate the true aflatoxin concentration among ears in a field. This is one of only a few studies that have been conducted to measure mycotoxin sampling statistics for field conditions.

Andersson *et al.* (2011) studied the use of manual and automatic sample selection methods to measure ochratoxin A (OTA) in barley grain. A nested experimental design was used to estimate variance components for sampling, sample reduction, sample preparation, and analysis. Results indicated that automatic samplers taking incremental samples from a moving stream can provide representative barley samples for OTA measurement.

Hallier *et al.* (2011) studied (a) the variability associated with sampling, sample preparation, and analysis steps used to test wheat grains for DON and (b) the effect of the degree of comminution (particle size) and extraction time on the quantification of DON in comminuted subsamples taken from the laboratory sample. Results showed that unlike flour, the wheat grain sampling step constituted the largest portion (about 46%) of the total variability of the DON testing procedure. The results demonstrated the importance of both sample size and of sample selection methods that provide representative samples, at reducing the variability associated with the sampling step. In the second study, the extraction time had a significant effect on DON extracted from the comminuted test portion. Using acetonitrile:water (84:16 v/v), significantly more DON was extracted using a magnetic agitator for 90 minutes than using a blender at high speed for 5 minutes (283.2 vs. 43.8 ng/g, respectively). Also, particle size had an effect on the efficiency of extracting DON from wheat. Contrary to what might be intuitively expected, extraction efficiency was higher for larger particle sizes (particles that passed a 2 mm screen, but rode a 0.5 mm screen) than smaller particle sizes (particles that passed a 0.5 mm screen, but rode a 0.05 mm screen). The authors attributed this result to there being a greater proportion of bran in the larger particle size.

Whitaker *et al.* (2010) published a manual with the help of the International Atomic Energy Agency (Vienna, Austria), that provides background information on methods to design effective mycotoxin sampling plans for food and feeds. The authors discuss uncertainty, both bias and variability, associated with a mycotoxin test procedure (sampling, sample preparation, and analysis) and how to reduce bias and variability in order to reduce buyer and seller risks (bad lots accepted and good lots rejected, respectively) associated with a mycotoxin sampling plan design.

3. Aflatoxin M_1

As compared to the period 2009-2010 (Shephard *et al.*, 2011), ELISA remained the main analytical technique in the monitoring studies for aflatoxin M_1 (AFM_1) in milk and milk products, followed by high performance liquid chromatography (HPLC), thin layer chromatography (TLC) and fluorometry (Figure 1). Whereas the application of TLC was applied only in 3 studies (Fallah, 2010; Fallah *et al.*, 2011; Filazi *et al.*, 2010), it is interesting to note that this cheap and simple veteran technique, in its two-dimensional format in combination with traditional silica gel column chromatography clean-up, was reported to be able to reach detection limits as low as approximately 0.012-0.020 µg/kg. These levels can compete with those reached with today's sophisticated methods, and are suitable for meeting regulatory requirements. Some publications have appeared on the determination of AFM_1 in milk and milk products with advanced and newer techniques such as HPLC-tandem mass spectrometry (HPLC-MS/MS) (Aguilera-Luiz *et al.*, 2011; Beltran *et al.*, 2011; Wang *et al.*, 2010, 2011a) and sensor methodology (Nivarlet *et al.*, 2011; Paniel *et al.*, 2010), yet these papers focused on method development and method optimisation rather than on use in routine operations.

The development of multi-analyte HPLC-MS/MS methods for the determination of mycotoxins and other possibly co-occurring residues and contaminants has also found first applications in the area of milk analysis, where chloramphenicol (Wang *et al.*, 2011a) and pesticides (Aguilera-Luiz *et al.*, 2011) can be determined simultaneously with AFM_1. The method development for chloramphenicol and AFM_1 was based on earlier HPLC-MS/MS work of the same authors focusing solely on the determination of AFM_1 (Wang *et al.*, 2010), and

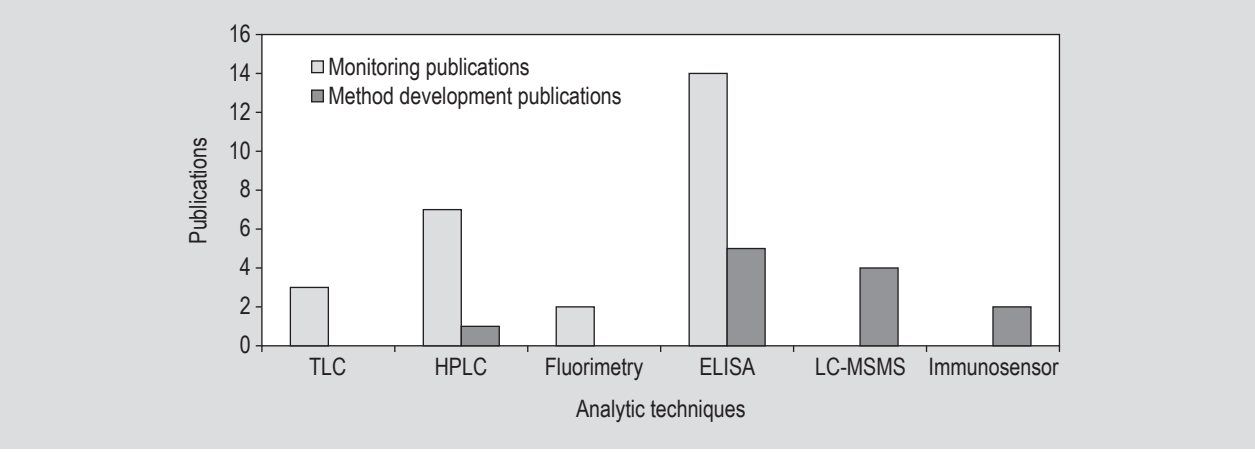

Figure 1. Frequency of analytical techniques used in papers on monitoring and occurrence of aflatoxin M_1 in milk and dairy products (indicated as monitoring publications) and in papers on method development (indicated as method development publications)

involves simple extraction directly followed by liquid chromatography (LC) and tandem mass spectrometry (MS/MS). The multi-method of Wang *et al.* (2010) was validated in-house according to Commission Decision 2002/657/EC (EC, 2002) and yielded a limit of quantification for AFM_1 at 0.02 µg/kg milk. Recoveries were reported between 90% and 97%, at levels ranging from 0.025-0.075 µg/kg, which is in the order of magnitude of the (stringent) EU regulations (EC, 2006). The multi-method for pesticides and AFM_1 is based on extraction by solid phase extraction (SPE) using C_{18} as sorbent and methanol. Final determination is performed by ultra-high performance liquid chromatography (UHPLC)-MS/MS. In-house validation showed a limit of quantification for AFM_1 at 0.03 µg/kg. Recoveries were determined at levels ranging from 0.05-2.50 µg/kg (the latter being unrealistically high, in respect of current regulatory levels for AFM_1), and were reported to range from 84% to 97%.

Immunosensor-based methods continue to be developed for the determination of AFM_1, although on a modest scale. Some developments have also taken place in the related area of ELISAs. Paniel *et al.* (2010) described the development of an electrochemical biosensor for the detection of AFM_1 in milk, based on a competitive immunoassay that makes use of magnetic beads, coated with anti-AFM_1 antibody, and screen-printed electrodes. The method is not a new development, but rather another variant of the sensor methodology, about which mention was made in the AFM_1 reviews in the periods 2008-2009 (Shephard *et al.*, 2010) and 2009-2010 (Shephard *et al.*, 2011). Small-scale or full-scale interlaboratory validation studies of sensor-based methods have not taken place yet. Nivarlet *et al.* (2011) presented a rapid dipstick test for the determination of AFM_1 in milk. This semi-quantitative test makes use of gold-labelled antibodies that compete between AFM_1 present in the milk sample and the toxin immobilised on the dipstick. When the sample is contaminated with AFM_1, the antibodies can no longer recognise the immobilised antibody and no colour will be generated at the test line. The test does not require any pre-treatment or cleaning of the milk and gives a semi-quantitative response after 20 minutes of incubation when the strips are analysed with an optical reader. Negatives (0-0.05 µg/kg), low positives (0.05-0.10 µg/kg) and full positives (0.05-0.10 µg/kg) can be distinguished. Another strip-format test for AFM_1 determination was developed by Wang *et al.* (2011b). With this test the whole analysis procedure of milk can be completed within 10 minutes, but the reported relatively high limit of detection (LOD) of 1 µg/kg drastically limits its use in practice.

Papers on ultra-sensitive ELISAs to determine AFM_1 in milk and infant milk products were published by Guan *et al.* (2011) and Kanungo *et al.* (2011). Detection limits as low as 0.003 µg/kg and 0.005 µg/kg, respectively, and recovery percentages around 100% were reported for both methods. It is interesting to note that the detection limits could be reached by applying the methodology simply on centrifuged milk, without the need for special clean-up. This is partly thanks to the use of sensitive magnetic nanoparticles, which have a high surface area and provide an enhanced number of binding sites. The reported low limits of detection are normally not needed, but may be useful for the analysis of milk used for infant foods, for which there are sometimes special requirements as to the level of contamination with AFM_1. The method of Kanungo *et al.* (2011) makes use of 384-well plates which allows high-throughput analyses, although the assay time is 4-5 hours, which is relatively long, if compared with the response-time of the dipstick method of Nivarlet *et al.* (2011) described above.

Articles on the occurrence of AFM_1 in milk and milk products continue to be published in abundance. In the period mid-2010/mid-2011 most articles on monitoring and surveillance came from Asia, in particular Iran (Fallah, 2010; Fallah *et al.*, 2011; Mahsa, 2010; Mohamadi and

Alizadeh, 2010; Mohamadi Sani *et al.*, 2010; Sefidgar *et al.*, 2011) and Turkey (Aksoy *et al.*, 2010; Aydemir Atasever, 2010a,b,c; Çaglar and Kara, 2010; Er *et al.*, 2010; Filazi *et al.*, 2010; Mutlu *et al.*, 2010; Ozsunar *et al.*, 2010). One of the Turkish studies involved the analysis of 75 samples of human breast milk (Gürbay *et al.*, 2010); in all samples low concentrations of AFM_1 were found. This pointed at the exposure of mothers and neonates to AFB_1 and AFM_1. Published surveys of milk and milk products carried out in other countries involved Brazil (Becker *et al.*, 2010), Egypt (Amr Amer *et al.*, 2010), Sri Lanka (Pathirana *et al.*, 2010), South Korea (Kim *et al.*, 2010), Spain (Cano-Sancho *et al.*, 2010a; Gómez-Arranz and Navarro-Blasco, 2010) and Thailand (Ruangwises and Ruangwises, 2010). In general, the contamination of milk and milk products with AFM_1 was low and in those cases where regulatory limits were exceeded, these breaches were often marginal and should not be a cause for concern. The statements of some of the authors of the monitoring studies that the results 'could be a serious public health problem' and that 'the high AFM_1 level is an important problem, threatening the public health' seem therefore exaggerated.

4. Aflatoxins B_1, B_2, G_1 and G_2

New and improved methods for aflatoxin determination using a range of analytical techniques continue to be published. Existing methods have been reviewed by Reiter *et al.* (2009), whereas Buttinger (2010) has surveyed interlaboratory studies spanning the previous 20 years and concluded that there has been no improvement in analytical performance by expert laboratories.

A matrix solid-phase dispersion (MSPD) method was developed in which a mixture of C_{18} sorbent and cereal sample was extracted by passing acetonitrile through the mixture contained in a glass column (Rubert *et al.*, 2010). Subsequent aflatoxin and OTA quantification was by HPLC-MS/MS. A similar method was used to determine aflatoxins in tiger nuts and their beverages by HPLC with fluorescence detection (HPLC-FLD), the mixture of sample and sorbent being initially extracted with hexane (Sebastia *et al.*, 2010). The use of a QuEChERS-based (quick, easy, cheap, effective, rugged and safe) extraction was compared with accelerated solvent extraction (ASE) for determination of 17 mycotoxins, including aflatoxins, by HPLC-MS/MS (Desmarchelier *et al.*, 2010). The two methods were found to give similar performances, but the former was easier to handle and allowed a higher sample throughput. A QuEChERS-based extraction has also been shown to be effective for analysis of aflatoxins and other mycotoxins in eggs using MS/MS for detection (Frenich *et al.*, 2011). A more conventional approach was followed by Campone *et al.* (2011a) in the determination of aflatoxins in cereal products in which the methanol/water extractant was partitioned with chloroform and additional water. Following centrifugation, the lower chloroform layer was dried down and aflatoxins determined by HPLC-FLD. Recoveries were between 67-92%.

Despite the rapid advance in LC-MS methods for aflatoxins, the year under review has also seen publications on new applications for conventional HPLC-FLD. Aflatoxins were determined in olive oil, peanut oil and sesame oil by extraction with methanol/water (55:45), followed by immunoaffinity column (IAC) clean-up and determination by HPLC with post-column UV photochemical derivatisation (Bao *et al.*, 2010). Recoveries ranged from 87.7 to 102.2%. A similar method, but with methanol/water (80:20) extraction, was optimised for aflatoxins in cassava flour and gave recoveries of 52-89% (Gnonlonfin *et al.*, 2010). Afsah-Hejri *et al.* (2011) performed an optimisation study for fluorescence response after post-column photochemical derivatisation of AFB_1, aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1) and aflatoxin G_2 (AFG_2) extracted from peanut and separated on a reversed-phase column with acetonitrile/water/methanol mixtures. Highest peak areas were obtained with a mobile phase composition 8:54:38 at 24 °C and flow rate of 0.4 ml/min. Under the conditions tested, the flow rate, as might be expected, had the greatest influence. A notable feature of the current review period was the number of publications reporting the determination of aflatoxins simultaneously with other mycotoxins. Soares *et al.* (2010) reported the use of reversed-phase HPLC combined with post-column photochemical derivatisation to separate the aflatoxin B series from the G series and cyclopiazonic acid extracted from *Aspergillus* species grown on agar medium. The mobile phase was methanol/4 mM $ZnSO_4$ (65:35) at pH 5. Brera *et al.* (2011a) conducted a single laboratory validation study (SLV) for the determination of aflatoxins and OTA in baby foods and paprika. Aflatoxins and OTA were extracted with methanol/water (80:20) and cleaned-up on a single IAC containing antibodies for both mycotoxins. Separation was performed on a C_{18} reversed-phase column with gradient elution. Aflatoxins were derivatised by post-column addition of pyridine hydrobromide perbromide and excitation and emission wavelengths for fluorescence detection were programmed to change after elution of the aflatoxins prior to OTA elution. Method validation parameters met current EU requirements. A method using acetonitrile/water extraction and clean-up on a multi-functional IAC for simultaneous quantification of aflatoxins, OTA and ZEA in barley was validated (Ibanez-Vea *et al.*, 2011a). Another research group has optimised a method for simultaneous aflatoxin, OTA and ZEA separation by HPLC (Rahmani *et al.*, 2011). Conditions for this separation were optimised with respect to column temperature (40 °C), flow rate (1 ml/min), aqueous phase acid concentration (0.1% acetic acid) and gradient composition (moving from 41% organic to 60% organic phase). A method for determination of aflatoxins, OTA and ZEA in cereals was validated (Rahmani *et al.*,

2010). Samples were extracted with methanol/water (80:20) and cleaned-up on a multifunctional IAC. Chromatographic separation was performed on a C_{18} reversed-phase HPLC column with gradient elution. Fluorescence detection was achieved with post-column photochemical derivatisation and time-based programming of the requisite wavelengths. Method validation parameters were reported to be within current EU requirements. This method has been further developed to encompass the above mycotoxins as well as DON, fumonisins, T-2 and HT-2 with double extraction, multifunctional IAC clean-up and suitable post-column derivatisation for aflatoxins and fumonisins (Soleimany *et al.*, 2011). The method required both fluorescence and UV detectors and for AFB_1 gave a LOD of 0.025 µg/kg and a mean recovery of 83% and was applied to cereal samples from the Malaysian market.

HPLC-MS/MS determination of aflatoxins, frequently in combination with other mycotoxins, has been widely reported. Li *et al.* (2011a) and Leong *et al.* (2011) reported the use of HPLC-MS/MS for determination of the four aflatoxin analogues in animal feeds, and commercial nuts and nut products, respectively. The former used stable isotope dilution to eliminate the extract clean-up step, whereas the latter used IAC clean-up and AFM_1 as an internal standard added to the test sample. Under the HPLC conditions with a fast gradient, AFM_1 co-eluted with AFG_2. Recoveries ranged from 76% to 101%, indicating little signal suppression. Other authors have combined aflatoxin determination with OTA determination. Barricelli *et al.* (2010) reported the determination of these mycotoxins in paprika and chilli powder using HPLC-MS/MS following extract clean-up on an IAC containing antibodies for both aflatoxin and OTA. Beltran *et al.* (2011) analysed baby food and milk for the above mycotoxins, as well as AFM_1. Samples were extracted with acetonitrile/water (80:20) and concentrated by using an IAC for clean-up. Two research groups have published the development and validation of HPLC-MS/MS methods for the determination of aflatoxins and other mycotoxins (apart from fumonisins) subject to European Commission regulation (Capriotti *et al.*, 2010; Lattanzio *et al.*, 2011). The former extracted wheat and maize by ultrasonication using an extractant of acetone/water/acetic acid (80:19:1). An aliquot of the extract was dried down, reconstituted in methanol/water/acetone (10:80:10) and analysed by HPLC-MS/MS with atmospheric pressure photoionisation. Only moderate signal suppression was found. Lattanzio *et al.* (2011) used acetonitrile/water (84:16) to extract cereal-based foods and a polymeric SPE column for clean-up prior to LC-ESI-MS/MS with fully ^{13}C-isotope-labelled internal standards. Other papers have described the development of an UHPLC-MS/MS method for 26 mycotoxins in teas and herbal infusions (Monbaliu *et al.*, 2010a), a HPLC-MS/MS method for 25 mycotoxins in cassava flour, peanut cake and maize (Ediage *et al.*, 2011) and an UHPLC method for 32 mycotoxins in beer using orbitrap-MS or TOFMS (Zachariasova *et al.*, 2010). Vaclavik *et al.* (2010) reported poor ionisation efficiencies for aflatoxins under direct analysis in real time (DART) ionisation. Although most publications involve food or feed items, Santini *et al.* (2010) have described a method for analysis of the 4 main aflatoxin analogues as well as their mammalian metabolites, AFM_1 and aflotoxin M_2 (AFM_2), in human sera. Schenzel *et al.* (2010) analysed for 33 mycotoxins in drainage water, river water and wastewater treatment plant effluent at a research site in Switzerland. Mycotoxins were isolated on Oasis® HLB cartridges prior to LC-ESI-MS/MS. No aflatoxin was detected, although a number of *Fusarium* toxins (BEA, DON, NIV, 3-acetyl-DON and ZEA) were reported. It is unsurprising that the mycotoxins reported in water are hydrophilic.

A novel multimycotoxin method (for aflatoxins, OTA, DON, fumonisins, ZEA and T-2) has been developed based on the MultiAnalyte Profiling (xMAP) technology, in which an analyte-bovine serum albumin (BSA) complex is coupled on the surface of a unique colour-coded paramagnetic microsphere suspension (Peters *et al.*, 2011). The specific spheres and monoclonal antibodies are added to the test solution containing mycotoxins, resulting in a competitive assay for available specific antibody. After magnetic capture, the sphere-bound antibody is detected by a fluorescent-labelled anti-antibody and subsequent counting in a dedicated flow cytometer. A full description has been published of the development and optimisation of a quantitative lateral flow immunoassay for total aflatoxins in maize (Anfossi *et al.*, 2011). The lateral flow device has a LOD of 1 µg/kg and dynamic range of 2-40 µg/kg. Two commercial systems (an ELISA for total aflatoxin in maize and an IAC for determination of total aflatoxins in peanuts and maize either by HPLC or solution fluorometry) have been validated as AOAC International Performance Tested Methods (Lupo *et al.*, 2010, 2011). A novel latex agglutination inhibition reaction test was developed for AFB_1 and tested as a rapid screening method in rice and peanuts (Ye *et al.*, 2011). Agglutination of AFB_1-labelled latex particles and anti-AFB_1 antiserum was inhibited by 5 ng/ml AFB_1. Zhang *et al.* (2011a,b) developed immunochromatographic assays in strip format using newly derived monoclonal antibodies for qualitative screening of either total aflatoxins or AFB_1 in peanuts. Other immunoassay methods published include a microplate ELISA with chemiluminescence detection (Fang *et al.*, 2011), an amperometric biosensor using carbon nanotubes as support for aflatoxin oxidase (Li *et al.*, 2011b) and a novel fluorescence immunoassay using AFB_1-functionalised magnetic beads and silica nanoparticles doped with rhodamine B fluorophore and carrying surface-bound anti-AFB_1 antibodies (Tang *et al.*, 2010).

Apart from the advances in HPLC and immunological methods above, a number of other techniques have

featured in new publications. The use of β-cyclodextrins in the mobile phase for TLC separation of aflatoxin and ZEA standards has been investigated (Larionova *et al.*, 2011). Micellar electrokinetic capillary chromatography with laser-induced fluorescence detection was used for determination of aflatoxins in rice (Arroyo-Manzanares *et al.*, 2010). Repeatabilities were less than 12% and recoveries between 93 and 105%. Multispectral and hyperspectral imaging have been shown to be effective in classifying maize kernels, hazelnut kernels and red chilli flakes with the aim of removing aflatoxin-contaminated units from a contaminated batch of agricultural product (Kalkan *et al.*, 2011; Yao *et al.*, 2010).

5. *Alternaria* toxins

The review of the 10 selected papers on *Alternaria* mycotoxins published in the period mid-2010/mid-2011 revealed that LC-MS(/MS) was the most applied technique (8 papers) followed by enzyme immunoassay (2 papers). Ackermann *et al.* (2011) reported the production of polyclonal and monoclonal antibodies against alternariol (AOH), and their implementation in enzyme immunoassay for the rapid determination in foods. The limits of detection were 35 ng/l for monoclonal antibody and 59 ng/l for polyclonal antibody, being in food products equivalent to 1 and 2 µg/kg, respectively. At these limits of detection AOH was found at levels of 1-13 µg/kg with high frequency in apple (67% of 44 samples) and tomato (93% of 44 samples) products. An indirect ELISA, based on polyclonal antibodies for AOH, was developed and used to investigate the occurrence of this toxin in oats, barley and animal feeds (Burkin and Kononenko, 2011). The limit of quantification was 0.4 µg/l, which is equivalent to 20 µg/kg in cereals and feeds. Average levels of positive samples were 144 µg/kg for oats, 48 µg/kg for barley and 23-760 µg/kg for animal feeds.

The presence of AOH and its monomethyl ether (AME) at levels of 37-71 µg/kg and 5-174 µg/kg, respectively, was reported by Wagacha *et al.* (2010) in 27% of 26 wheat samples collected in Kenya. The samples were extracted with an acetonitrile/water/acetic acid mixture and directly injected into a HPLC-MS/MS system without any dilution procedure. [$^{13}C_6$,^{15}N]-tenuazonic acid was synthesised and used as internal standard in the HPLC-MS/MS determination of tenuazonic acid in tomato products after derivatisation with 2,4-dinitrophenylhydrazine (Asam *et al.*, 2011a). This approach is also known as stable isotope dilution assay (SIDA). LOD and limit of quantitation (LOQ) were 0.1 and 0.3 µg/kg, respectively, whereas the repeatability was 2-5%. Values between 15 and 195 µg/kg, 363 and 909 µg/kg and 8 and 242 µg/kg were measured in commercial samples of tomato ketchup, tomato paste and pureed tomatoes, respectively. An existing SIDA for AOH and AME determination in grape and vegetable juices was applied to cereal products and vegetable products (Asam *et al.*, 2011b). Both toxins were practically not detected in cereals and cereal products whereas cereals for animal nutrition were found to be contaminated at mean levels of 78 µg/kg for AOH and 28 µg/kg for AME. Low mean levels of AOH (4.3 µg/kg) and AME (0.7 µg/kg) were measured in vegetable, mainly tomato, products. AOH was found in 6 out of the 20 samples of commercial sweet peppers that showed signs of fungal spoilage (Monbaliu *et al.*, 2010b). The levels varied from below the LOQ (6.6 µg/kg) to 101 µg/kg. The multi-mycotoxin method used for determination of AOH, AME, altenuene (ANE) and another 21 mycotoxins involves a double extraction with an ethyl acetate/formic acid mixture and the use of 3 columns (C_{18}-SPE, SAX and NH_2-SPE) for sample extract clean-up before HPLC-MS/MS determination.

An UHPLC-orbitrap-MS multi-mycotoxin method was developed for determination of 32 mycotoxins in beer, including AOH and AME (Zachariasova *et al.*, 2010). The beer sample was diluted with acetonitrile to precipitate matrix components, centrifuged and the supernatant dried, reconstituted in methanol/water (50:50), and analysed. Matrix-matched standard calibration curves were used to compensate for matrix effects. The lowest calibration levels of matrix-matched standard AOH and AME were 1.5-2.0 and 1.0-3.0 µg/l, respectively, whereas recoveries ranged from 96 to 107% for AOH and 92 to 113% for AME. Repeatability ranged from 16 to 29% for AOH at level of 0.5 µg/l and from 14 to 23% for AME at level of 1 µg/l. UHPLC-orbitrap-MS was also used for the analysis of 24 mycotoxins, including AOH, AME and ANE, in extracts of wheat, maize and millet purified with a modified QuEChERS procedure (Vaclavik *et al.*, 2010). In this case higher values of the lowest calibration levels were reported, i.e. 50, 80 and 90 µg/kg for AOH, AME and ANE, respectively.

Low recoveries (30-50%) were reported for AOH and AME in a multi-residue HPLC-MS/MS screening method developed to quantify 33 mycotoxins in drainage, river and wastewater treatment plant effluent water (Schenzel *et al.*, 2010). Degradation products of AOH and AME (6-methylbiphenyl-2,3',4,5'-tetrol and 5'-methoxy-6-methylbiphenyl-2,3',4-triol), formed upon bread baking, were synthesised and identified at low levels (<45 µg/kg) in commercial cereal-based baking products together with low levels of AOH and AME. A new HPLC-MS/MS method was developed for simultaneous determination of AOH, AME, ANE and the two degradation products of AOH and AME. No degradation products of ANE were found (Siegel *et al.*, 2010). An EFSA scientific opinion on the risk for animal and public health related to the presence of *Alternaria* toxins in feed and food is expected to come out soon.

6. Ergot alkaloids

The determination of ergot alkaloids continues to arouse interest with publications describing modifications to analytical methods and a range of applications. One pleasing development has been that analytical standards including epimers are appearing on the commercial market, along with rudimentary reference materials, although isotopically labelled internal standards useful in LC-MS quantification are still unavailable.

The third part in a very concise history of ergot has been provided by Lee (2010). It covers the period 1940 to 1980 and is restricted to the well-reported discovery of the effects of lysergic acid diethylamide and the pharmaceutical uses of bromocryptine. An important and useful review of ergot related disease in cattle has been published by Strickland *et al.* (2011). It introduces the ergot fungi and briefly discusses the natural sources of the alkaloids. A tabular list of analytical methods is structured by alkaloid and source fungus with an emphasis on methods for animal tissues and fluids. However, some methods for food analysis that could readily be applied to body tissues are omitted. The major part of the review gives valuable and detailed information on the metabolism of the alkaloids. Further descriptions of the effects of ergopeptides on pregnant mares have been provided by Evans (2011). Another brief description of the importance of ergot in livestock production was prepared by Pedrosa and Griessler (2010).

A new peptide ergot alkaloid, *N*-(*N*-lysergyl)-cyclo(isoleucyl-phenylanalyl-prolyl) or ergosedmine, and its C8 epimer, have been isolated by Uhlig *et al.* (2011) from the sclerotia of *Claviceps purpurea* infecting feather reed grass (*Calamagrostis arundinacea)*. The alkaloid was found in much lower abundance than the major familiar alkaloids. Ergot poisoning of water buffaloes was reported in the UK (Millar *et al.,* 2010). The ergot was identified as sclerotia in grass silage after clinical signs of illness did not indicate a different cause. In a similar case, infected seed heads indicated the poisoning of horses by *Claviceps paspali* in Australia (Cawdell-Smith *et al.,* 2010).

A few surveys of the occurrence of mycotoxins included ergot alkaloids. Liesener *et al.* (2010) determined multi-mycotoxins in 62 commercial samples of horse feed by ELISA that included generic ergot alkaloids. The method was developed in-house using antibodies to ergonovine and had cross reactivity towards all ergoline alkaloids, providing a generic total ergot alkaloid result. The detection limit for ergot alkaloids was 30 µg/kg, with a rather high recovery (132%). Ergot alkaloids were detected in about 60% of samples at levels between 28 and 1,200 µg/kg. The method included a simple extraction with acetonitrile/phosphate buffered saline (PBS) followed by centrifugation and competitive direct ELISA using antigens developed from ergonovine. Reinhold and Reinhardt (2011) surveyed multi-mycotoxins in 500 retail food samples in Germany using HPLC-MS/MS, but ergot determination was restricted to 31 rye flours, of which about 50% proved positive. 90% of the samples contained <500 µg/kg total alkaloids with a mean value of 213 µg/kg. The highest result was just over 1000 µg/kg. A multi-mycotoxin analysis of cereal grains carried out by Martos *et al.* (2010) determined 22 mycotoxins including ergotamine and ergocornine. Toxins were extracted into acetonitrile:water using a Stomacher® paddle blender that kneads the solvent/sample mixture in a bag. Extraction was completed in 2 min. The method was validated in-house with respect to matrix effects, accuracy, repeatability and ruggedness and applied to 100 samples of wheat, maize, oats, barley and rye. The extracts were centrifuged and analysed by HPLC-MS/MS with mini-column HPLC giving a run time of 5 min. Detection limits were in the order of 10 µg/kg and varied slightly with matrix. Epimerisation was prevented by the choice of extraction solvent but any natural epimeric content was not reported, as the choice of solvent possibly failed to allow separation of epimer peaks. Ergot alkaloids were found only in wheat. Ergotamine was reported in one winter wheat (638 µg/kg) and three spring wheats (88-146 µg/kg). Ergocristine was found in a different winter wheat (76 µg/kg) and in three spring wheats (89-354 µg/kg) one of which did not contain ergotamine. Presumably the method could be adapted easily to include other ergot alkaloids.

Two other publications described rapid HPLC-MS/MS methods. Kokkenen and Jestoi (2010) developed a method to determine 6 ergot alkaloids and 4 epimers in rye and wheat. The alkaloids were extracted into acetonitrile/aqueous ammonium carbonate solution and purified with a commercial SPE column designed for this analysis (Mycosep® 150 Ergot). The quantification limits were <1 µg/kg for wheat and <10 µg/kg for rye. Matrix effects necessitated the use of matrix matched standards for quantification. Zachariasova *et al.* (2010) measured 5 of the major ergot alkaloids and their epimers (not ergotamine/ergotaminine) in a suite of 32 mycotoxins in beer using ultra high resolution mass spectrometry following a simple extraction. Matrix compounds were precipitated by adding acetonitrile and the solution was analysed by DART-MS. The method had good recoveries and detection limits for all analytes. Again, matrix matched standards were recommended, along with isotopically labelled internal standards where available.

The distribution of ergot alkaloids in milled grain fractions was studied by Franzmann *et al.* (2011) for a model milling process. The analytical methods were based on determination of both the alkaloids and ricinoleic acid, a characteristic fatty acid of ergot oil. Determination of the alkaloids used HPLC-FLD after an alkaline organic extraction. Methylsergide was used as an internal standard.

Both methods had been reported previously (Franzmann *et al.*, 2010). The results showed that ergot sclerotia should be removed as early as possible from bulk cereals and that abrasion should be reduced in order to minimise alkaloid transfer to grain surface. The alkaloid concentration measured in the product was higher in the finer product fractions. Analysis of 63 sclerotia showed them to have an average alkaloid content of about 750 µg/kg, which is within the ranges reported earlier (Mainka *et al.,* 2007; Wolff, 1989).

There is growing interest in the fate of many natural toxins in composted plants, hay and silage, and studies have been applied to ergot infected materials. Siegrist *et al.* (2010) showed that levels of ergot alkaloids in tall fescue grass infected with *Neotyphodium* decreased rapidly when the grass decomposed in litter bags, which suggests that these alkaloids do not inhibit decomposition of composted fescue grass. Alkaloids were measured by the method of Yates and Powell (1988) based on HPLC-FLD. Blaney *et al.* (2010) showed that ensilage reduced dihydroergosine from *Claviceps africana* by 50% over four weeks. The alkaloid was measured by an ELISA method (Molloy *et al.,* 2003).

ELISA procedures have also continued to find favour. Hopkins *et al.* (2010) reported that ELISA analysis of contaminated fescue grass gave overly high results on account of a background effect in some samples. This was attributed to the presence of setoclavine, an oxidation product of agroclavine. The ELISA procedure devised by Hill and Agee (1994) was used by Rogers (2010a,b, 2011) to determine total ergot alkaloids in fescue forage samples. Comprehensive experiments are described (Rogerss, 2010a) regarding field and crop management techniques to reduce ergovaline and ergot alkaloid concentrations in forage.

Studies on the cytoxicity of ergot alkaloids were carried out by Mulac and Humpf (2011). The peptide alkaloids, especially ergocristine, were the most toxic. The alkaloids were determined using HPLC-FLD. Different mobile phase programmes were used for the peptide alkaloids and to separate the ergometrine/ergometrinine pair. Methysergide maleate was used as an internal standard. Stability tests in cell medium allowed the assignment of stability factors for each epimeric pair. Ergometrine and ergosine were the most resistant to epimerisation.

7. Fumonisins

HPLC continues to be widely used for separations, although use of UHPLC, with the potential for reduced separation times, continues to expand in use. The fumonisins lack a good chromophore. Therefore chromatographic methods for their detection generally rely upon mass spectrometry, tandem mass spectrometry or derivatisation and detection by fluorescence or UV absorbance. The use of fluorescent labels, particularly *o*-phthaldialdehyde (OPA) is well established and, in the past year several papers have further examined this approach (Belajová and Rauová, 2010; De Girolamo *et al.*, 2011a; Oles and Trucksess, 2010; Soleimany *et al.*, 2011; Solfrizzo *et al.*, 2011). Two SLVs of methods incorporating derivatisation with OPA were reported (Belajová and Rauová, 2010; Oles and Trucksess, 2010). In the first of these a pre-column derivatisation method was validated for use in botanical roots (Oles and Trucksess, 2010). A mixture of methanol/acetonitrile/water (25:25:100) was used for extraction, prior to clean-up with an IAC. Average recoveries ranged from 67 to 100%. Total relative standard deviations (RSDs) for within-day and between-day repeatability ranged from 5.5 to 26.4%. All of the botanicals that were examined contained less than 10 µg/kg of fumonisin B_1 (FB_1). The second SLV study was of a pre-column OPA derivatisation method for FB_1 and fumonisin B_2 (FB_2) in cereals and cereal-based foods (Belajová and Rauová, 2010). Average recoveries ranged from 87 to 93% (FB_1) and 83 to 96% (FB_2). Fluorescence detection has also been combined with photodiode array (PDA) detection to allow multiple analysis of 12 mycotoxins (Soleimany *et al.,* 2011). The method uses a double extraction, first with PBS, then with methanol. Extracts were cleaned up using a multifunctional IAC (AOFZDT2; Vicam, Watertown, MA, USA). The HPLC system incorporated a PDA detector (to detect DON, T-2 and HT-2), a photochemical reactor for enhanced detection (PHRED) system (for the aflatoxins), a post-column derivatisation apparatus (PCX5200; Pickering Laboratories, Mountain View, CA, USA, for the fumonisins by OPA), and a fluorescence detector. Average recoveries from spiked maize ranged from 79-93% (FB_1), 81-102% (FB_2), and 73-100% (fumonisin B_3; FB_3). The method was used to test 45 samples of rice, wheat and maize from Malaysia where fumonisins were found in relatively low levels (below 81 µg/kg). A validation study has been successfully carried out for determination of FB_1 and FB_2 in maize-based infant foods (Solfrizzo *et al.*, 2011). The method involved warm extraction with citrate phosphate buffer/methanol/acetonitrile (50:25:25), IAC clean-up and automated precolumn derivatisation with OPA. HorRat values were 0.8 to 1.4. Pre-column derivatisation with OPA was also used in two reports examining extraction and clean-up procedures for isolating fumonisins from masa flour (De Girolamo *et al.,* 2011a) or rice (Awaludin *et al.,* 2009). The first of these examined extraction from yellow, white, and blue masa flours, comparing acidic extraction with the use of EDTA (De Girolamo *et al.,* 2011a). They also compared isolation using C_{18}-SPE with IAC. The optimised method involved extraction with a mixture of methanol/acetonitrile and citrate/phosphate buffer with clean-up by IAC. Overall recoveries from masa averaged 99% for FB_1 (±6%) and 88% for FB_2 (±6%). In examining masa flour and tortilla chips the authors found combined (FB_1+FB_2) levels of up to 1,800 µg/kg in masa flour and 960 µg/kg in tortilla chips. Awaludin *et al.* (2009) developed an in-

house reference material and used it to examine factors affecting the extraction of fumonisins from rice. Several solvent:sample ratios were examined. Submerging the rice in water for 30 min, before adding methanol to the extraction increased the levels found (Awaludin *et al.,* 2009). Khayoon *et al.* (2010) examined the use of a silica-based monolithic column (Chromolith® RP-18; Merck, Darmstadt, Germany), which shortened the chromatographic separation times of the fumonisins to about 4 min.

Of the new analytical methods reported in the past year, most involved some form of LC-MS or HPLC-MS/MS, which indicates their use for fumonisin analysis is becoming increasingly widespread. An interlaboratory study was published describing an LC-MS method for fumonisins in maize (Senyuva *et al.,* 2010). Valid data were obtained from 12 laboratories in 11 countries, some using LC-MS and some using HPLC-MS/MS. The method involved extraction of maize with a mixture of acetonitrile/methanol/water (25:25:50), clean-up by IAC and reversed-phase LC followed by selected ion monitoring. Of the participating laboratories, 11 obtained recoveries from spiked maize samples ranging from 81 to 138% (FB_1), 71 to 138% (FB_2) and 77 to 138% (total fumonisins). The values for repeatability for FB_1 and FB_2 were 4.6-11.9% and 1.9-12.6% and for reproducibility were 19.8-23.8% and 18.2-25.5%, respectively. HorRat values were all below 2.0 for both repeatability and reproducibility. These performance characteristics suggest the method is suitable for use as a tool for regulatory enforcement of FB_1 and FB_2 in maize.

A large number of HPLC-MS/MS methods for fumonisins either singly or in combination with other mycotoxins in multi-toxin methods have been recently reported. Given the large number of papers, we can only briefly summarise them here. These have been subdivided into those that focus primarily upon the fumonisins (Han *et al.*, 2010a; Li *et al.*, 2010; Ren *et al.*, 2011) and those that include fumonisins among a larger number of targets for analysis (Desmarchelier *et al.*, 2010; Ediage *et al.*, 2011; Martos *et al.*, 2010; Monbaliu *et al.*, 2010a,b; Sørensen *et al.*, 2010; Tamura *et al.*, 2011). Those dealing primarily with fumonisins were developed to detect these toxins in a variety of different matrices including cereal-based commodities (Li *et al.*, 2010; Ren *et al.*, 2011) and traditional Chinese medicines (Han *et al.*, 2010a). All these methods applied UHPLC for the separation. The two methods that were applied to cereal grains used either methanol/water (70:30) (Li *et al.*, 2010) or acetonitrile/water (1:1) (Ren *et al.*, 2011) for the extraction. Both used an isotopic internal standard. The two methods differed in clean-ups, with either a centrifugation step followed by filtration (Li *et al.*, 2010) or centrifugation and further clean-up by SPE (Ren *et al.*, 2011). The method of Li *et al.* was reported to be fast, with the procedure taking less than 30 min. A SLV of the procedure indicated recoveries of FB_1, FB_2, and FB_3 ranging from 93%-108% (Li *et al.*, 2010). Results corresponded nicely with the AOAC method 995.15 (an LC-FLD method) for analysis of four feed samples. Ren *et al.* (2011) also reported an SLV for their method, applied to maize. Recoveries for the three fumonisins ranged from 80.9-97.0% with RSD values of 2.4-11.1%. The method was used to examine maize from six cities in China, and the mean levels found were reported, with the highest mean level 2.78 mg/kg FB_1 (Ren *et al.*, 2011). The method of Han *et al.* (2010a) was applied to traditional Chinese medicines. Samples were extracted with aqueous acetonitrile (50%) and purified by SPE. The LOQs were reported to be 0.08 to 0.16 ng/ml, with recoveries of 88.2-113.3% and an RSD of less than 12.3%. The method was applied to 35 samples, 18 of which were found to contain fumonisins, although at relatively low levels.

Most of the HPLC-MS/MS methods that were reported in the past year were used to test for fumonisins along with other analytes, generally other mycotoxins. The nature of the other analytes and the food being tested can have a bearing upon the extraction, clean-up, and detection conditions. Because multi-toxin methods have been optimised for multiple analytes, the conditions may or may not be those that are optimal for detection of the individual toxins. This is not to say that the multi-toxin methods necessarily perform any worse than methods dedicated solely to the fumonisins, only that the performance characteristics need to be considered within this context. Recent reports of new multi-toxin HPLC-MS/MS methods include application to grains, cassava flour, peanut cake, meat, tea, herbal infusions, sweet peppers, and beer. Because of the high prevalence of fumonisins in maize there is still a significant focus on methods for grains. Three such reports include applications for 22 mycotoxins in wheat, barley, oats, rye and maize (Martos *et al.*, 2010), 17 mycotoxins in maize and maize gluten, wheat, rye, rice, oats, barley, soya, and infant cereals (Desmarchelier *et al.*, 2010), and 25 mycotoxins in cassava flour, peanut cake, and maize (Ediage *et al.,* 2011). Desmarchelier *et al.* (2010) developed two methods, one based upon a QuEChERS-like clean-up and the other using ASE. The extraction solvent used for the QuEChERS approach was a combination of water and 0.5% acetic acid in acetonitrile, followed by addition of a $MgSO_4$/NaCl mixture. The extraction solvent used for the ASE was acetonitrile/water/glacial acetic acid (80:19:0.5). Interestingly, the same salts used in the QuEChERS approach were also used to help purify the ASE extract. Both gave similar performances with regards to linearity, precision, and limits of detection. However, the QuEChERS method was described as being easier and allowed a higher sample throughput.

A modified QuEChERS procedure using acetonitrile with NaCl, $MgSO_4$, and sodium citrate was used in a method to quantify 15 mycotoxins in beer and beer-based drinks (Tamura *et al.*, 2011). Following extraction the mycotoxins

were isolated by SPE then analysed by UHPLC-MS/MS. Of 24 beer-based drinks in Japan, fumonisins were detected in several samples at levels below the LOQ (5 µg/l). A MSPD technique was used in the extraction for a multi-toxin HPLC-MS/MS method (Rubert *et al.*, 2011a). A wide variety of solid phases were examined, including C_{18}, C_8, celite, silica, florisil, phenyl, alumina, and amine-based materials. Of these the fumonisins were only extracted when C_{18} and C_8 were used, and C_{18} was selected for use in the optimised method that was applied to flours. UHPLC-MS/MS was also used by Monbaliu *et al.* (2010a) to test for multiple mycotoxins in raw tea and herbal infusion materials and their drinkable products. Of the 91 samples of tea, herbal infusions and drinkable products that were tested only one, a Ceylon mélange, contained FB_1 (at 76 µg/kg). The same group also applied HPLC-MS/MS to detect multiple mycotoxins in sweet peppers (Monbaliu *et al.*, 2010b). HPLC-MS/MS was also used in a method for the determination of OTA, mycophenolic acid and FB_2 in meat products (Sørensen *et al.*, 2010). No FB_2 was reported in the sausages or in 22 retail products that were tested.

In the past year a number of papers have appeared investigating the possible use of fumonisin in urine as a biomarker. Several have used HPLC-MS/MS to detect fumonisins in urine (Ahn *et al.*, 2010a; Rubert *et al.*, 2011b; Van der Westhuizen *et al.*, 2011; Xu *et al.*, 2010). Also reported were an LC-FLD method (Xu *et al.*, 2010), and an immunoassay method (Desalegn *et al.*, 2011).

While most of the activities for developing new methods for fumonisins have been concerned with chromatographic methods, as described above, there have also been advances in antibody-based assays (immunoassays and biosensors), as well as non-antibody-based detection technologies. Common immunoassay formats include both ELISAs and lateral flow devices (LFDs). Rabbit polyclonal antibodies (Pab) for the fumonisins were developed and applied in both ELISA and LFD formats (Shiu *et al.,* 2010). ELISAs based upon the antibodies were very sensitive for FB_1 and FB_2 with IC_{50}s of 0.42 and 0.58 ng/ml, respectively. ELISAs were less sensitive for FB_3 (81.5 µg/l). When applied in an LFD format using gold nanoparticles the LOD was 5 µg/l (Shiu *et al.*, 2010). A new quantitative LFD was reported with a LOD of 120 µg/l (Anfossi *et al.,* 2010b). In a comparison between the LFD and an HPLC-MS/MS method for 27 naturally contaminated samples of raw maize, maize feed, maize starch, and maize meal a linear correlation was observed (r^2=0.990; Anfossi *et al.,* 2010b). An ELISA for fumonisins was developed and used as the basis for the development of an electrochemical immunosensor. The ELISA obtained had a LOD of 100 µg/l (Kadir and Tothill, 2010). This past year there were also two reports of flow cytometer-based microsphere immunoassays (Anderson *et al.*, 2010; Peters *et al.*, 2011). The first report was for detection of OTA and FB_1 in maize and oats (Anderson *et al.*, 2010), while the second was for the detection of six groups of mycotoxins (aflatoxins, OTA, DON, fumonisins, ZEA, T-2) in feeds (Peters *et al.*, 2011). Both used the same fumonisin antibody and the same brand of flow cytometer (Luminex, Austin, TX, USA). They differ in the assay particulars such as the secondary antibodies (e.g. detection labels), number of toxins in multiplexed assays, and the food matrices examined. With both there were marked effects of matrix upon the sensitivities of the assays.

While antibodies are one type of binding material that are useful in fumonisin assays, other types of binding materials continue to be developed, including molecularly imprinted polymers (MIP) and aptamers. McKeague *et al.* (2010) isolated six single-stranded oligonucleotides (aptamers) with an affinity for immobilised FB_1. One of the aptamers bound to immobilised FB_1 with a dissociation constant of 100 nM. Two presumptive tests for fumonisin contamination were also developed. One of these was an electronic nose that was used to discriminate between maize cultures containing either high or low levels of fumonisins (Gobbi *et al.*, 2011). The second combined multispectral imaging and a neural network to predict fumonisin content in ground maize (Firrao *et al.*, 2010). A correlation (r^2=0.68) was demonstrated between the predictions from the image analysis and the concentration of fumonisin determined by LC-FLD.

8. Ochratoxins

HPLC-FLD and IAC clean-up continues to be the most frequently used technique in routine analysis for OTA in foods, as deduced from the reports issued by proficiency testing schemes. As an example, in a proficiency test (PT) conducted on OTA in paprika, 46 out of 62 participants used IAC clean-up and 8 participants used SPE, 48 participants used HPLC and only 4 participants used ELISA, and 45 participants used fluorescence detectors with only 5 participants using MS/MS detectors (FAPAS, 2010). Similar figures were reported in other PTs. So, in a PT on OTA in roasted coffee, 47 out of 61 participants used IACs and 9 used SPE, 45 participants used HPLC and only one used ELISA, and 46 participants used fluorescence detectors with only 3 participants using MS/MS detectors (FAPAS, 2011).

Dohnal *et al.* (2010) investigated the influence of composition and pH of the HPLC mobile phase on OTA fluorescence. They found that while the first maximum excitation wavelength at 230 nm was pH-independent, the second one at 335 nm changed in alkaline pH to 380 nm. In general, there was a decrease in the fluorescence intensity when increasing the acetonitrile content in the aqueous buffer/organic solvent mobile phase mixture. The lowest LOD was 0.3 µg/kg in cereal samples, which was comparable with results obtained by other authors who used pH 9.8 and 20 mM NH_4Cl/NH_3 buffer. That LOD

was reached with 83 mM potassium acetate solution at pH 9.0. Fabiani *et al.* (2010) compared three different clean-up procedures and two analytical techniques for determination of OTA in wine. They found that best recoveries were obtained with IACs whereas the worst recoveries were with liquid-liquid extraction. HPLC showed good precision and recovery, whereas ELISA generally underestimated the OTA content. Remiro *et al.* (2010) reported the validation of a HPLC method with IAC clean-up for the simultaneous detection of OTA and its analogues, ochratoxin B (OTB), ochratoxin C (OTC) and methyl OTA in red wine. The LOQ was 0.50 ng/l for all the analogues. Recoveries ranged from 73.4% for OTC to 93.5% for OTA, respectively. Reiter *et al.* (2011) presented a new sample clean-up method based on immuno-ultrafiltration followed by HPLC-FLD coupled to an electrochemical cell for the analysis of OTA in cereals. In immuno-ultrafiltration, antibodies were used in non-immobilised form and electrochemical cell eliminated matrix interferences by oxidation. The authors reported an improvement in the quality of the chromatograms when compared with other widely used clean-up techniques such as IAC and multifunctional columns. Recoveries were above 70% in three tested cereals with a LOQ (S/N=10) of 1 µg/kg. The use of corona discharge ion mobility spectrometry for the determination of OTA in liquorice root was investigated by Khalesi *et al.* (2011). This innovative approach yielded an acceptable detection limit of 0.01 ng injected and a good recovery. Although information was not given in the article for translation of that detection limit into concentration in analysed samples, its fast response, low cost, simplicity and portability, allowed the authors to propose this technique as a competitive alternative to other ones, at least for screening purposes. Another quite novel approach in the field of mycotoxin analysis was presented by Campone *et al.* (2011b) for the rapid analysis of OTA in wine. This procedure used dispersive liquid-liquid microextraction and HPLC-MS/MS with positive ESI. The LOQ was 0.015 µg/l, the accuracy when applied to the analysis of reference material was 103% and the precision was 5.8%.

As regards rapid visual tests, Bazin *et al.* (2010) published a review focused on membrane-based strip tests, flow-through tests and clean-up tandem immunoassay formed by a clean-up column connected to a flow-through column for OTA detection based on the direct competitive immunoassay principle. Yu *et al.* (2011) compared a newly developed direct competitive chemiluminescent ELISA with a conventional colorimetric ELISA, and found better detection properties in the new approach when it was applied to the analysis of 21 various agricultural commodities. Beloglazova *et al.* (2010) reported the development of a column test with three separate immunolayers for the simultaneous one step detection of OTA and 2,4,6-trichlorophenol in wine with a cut-off value of 2 µg/l for both analytes. A regenerable immuno-biochip with flow-through reagent addition and chemiluminescence detection carried out in an automated microarray chip reader for screening OTA in green coffee extract was developed by Sauceda-Friebre *et al.* (2011). The total analysis time was 12 minutes and the LOQ was 7 µg/kg. Fernández-Baldo *et al.* (2010) described the development and characterisation of an electrochemical method using square wave voltammetry combined with the use of modified magnetic nanoparticles for determination of OTA in grapes with a LOD of 0.02 µg/kg.

Aptamers are oligonucleotides or short peptides that can bind with high specificity to target molecules such as OTA. These aptamers are inexpensive and can be easily labelled with a variety of molecules such as enzymes, biotin, fluorescent dyes, etc. that enable the development of a variety of detection methods. The use of aptamers has been the subject of a number of papers published during the last year. Thus, Kuang *et al.* (2010) described the development of an aptamer-based electrochemical sensor and its successful application to wine at a sensing range from 0.1 to 20 µg/l. Its potential as an alternative technology in the analysis of OTA was discussed. Yang *et al.* (2011) presented an aptamer-based colorimetric biosensing of OTA with a LOD of 20 nM. Barthelmebs *et al.* (2011a) studied the performance of various aptamers and investigated the use of both direct and indirect competitive enzyme-linked aptamer assays (ELAA) for detection of OTA in wine. The selected aptamer with the best IC_{50} and LOD, combined with the use of direct ELAA, compared well with the classical directly competitive ELISA, but overcame some known disadvantages of antibodies. Given the binding properties displayed by that aptamer, the authors also suggested its potential use as a novel approach to remove OTA from foods and beverages. As a follow-up of that work, Barthelmebs *et al.* (2011b) reported the development of a novel electrochemical aptasensor based on competitive assay with a LOD of 0.11 µg/l. Bonel *et al.* (2011) also described the development of a competitive aptasensor for OTA based on a DNA biotinylated aptamer, coupled to paramagnetic beads with electrochemical detection, able to be used for the determination of OTA in wheat well below the maximum level allowed in the European Union for cereals (3 µg/kg). The LOD was 0.07 µg/l and negligible cross-reactivity of the aptamer with OTB was observed. Rhouati *et al.* (2011) reported the development of an oligosorbent used for selective extraction detection of OTA in beer as an alternative approach to the IAC clean-up. In this case, aptamers were immobilised on cyanogen bromide-activated Sepharose and its use showed even better performances than IACs in terms of reusability and selectivity. IAC exhibited greater variability in recovery rates and standard deviations. In addition, the decrease in the recovery rate was, at most, 5% for the oligosorbent, after three replicates. The decrease observed when re-using the IAC varied between 37 and 95% after three replicates. In this study, aptamers could be recovered in a few minutes, while

antibodies needed several days and their performances were lower even after recovery. The working range was 0.5-10 µg/l. Similarly, De Girolamo *et al.* (2011b) reported the good behaviour of a DNA aptamer conjugated to a coupling gel and used as sorbent for the preparation of SPE columns in the analysis of durum wheat samples. The results showed good correlation with those obtained when using conventional IACs. Further developments and optimisations in the use of aptamers were reported by Wang *et al.* (2011c) who described the fabrication of an aptamer-based chromatographic strip assay able to be used for the qualitative visual detection of OTA in wine at the level of 1 µg/l while the LOD of the semi-quantitative detection was 0.18 µg/l when using a scanning reader. Duan *et al.* (2011) investigated the optimal experimental conditions to achieve the best analytical performances in the application of an aptamer-based fluorescence assay for OTA in wine with a LOD of 10 µg/l. Sheng *et al.* (2011) showed the advantages of using polivinylpyrrolidone-coated graphene oxide instead of bare graphene oxide in terms of sensitivity, when using the carboxyfluorescein-modified aptamer approach for determination of OTA in wine. Alonso-Lomillo *et al.* (2011) summarised the manufacturing procedure of horseradish peroxidase-based biosensors for the determination of OTA by using the single technology of screen-printing, and Campás *et al.* (2010) overviewed the different techniques developed for the detection of both OTA and microcystins, as representative examples of toxic secondary metabolites, with special emphasis on biosensor-based strategies as powerful screening tools.

Once again, a number of papers reporting data on the presence of OTA in commodities from different countries were published this year. Although some of these studies were sometimes specifically designed as pure survey studies, they were often conducted to demonstrate the applicability of newly developed or validated analytical procedures. In any case, they constitute a source of useful information that continues to show the wide occurrence of OTA in certain foodstuffs and biological fluids. Thus, Duarte *et al.* (2011) reviewed the current knowledge on OTA biomarkers, also paying some attention to the debate on the existence of OTA-DNA adducts. Brera *et al.* (2011b) used a previously published analytical method based on IAC clean-up and HPLC-FLD for the determination of OTA in 300 samples of cocoa and chocolate products from the Italian market. The experimentally confirmed LOQ was 0.080 µg/kg and all 40 cocoa powder samples were found positive with a mean concentration of 0.55 µg/kg; 159 of the 260 chocolate-based products contained OTA with a mean value of 0.29 µg/kg. Turcotte and Scott (2011) carried out a study on the presence of OTA in cocoa and chocolate sampled in Canada using the same analytical procedure mentioned before, after introducing some modifications. These included some changes in the mobile phase and addition of 40% methanol to the aqueous $NaHCO_3$ extraction solvent in order to eliminate chromatographic interferences and to improve the sensitivity of the method by achieving a LOQ of 0.07-0.08 µg/kg. Analysis of 32 samples of cocoa powder showed an incidence of 100% with concentrations ranging from 0.25 to 7.8 µg/kg. Three widely used extraction procedures for OTA in roasted coffee were compared and discussed by Tozlovanu and Pfohl-Leszkowicz (2010). The authors stated that OTA in coffee was frequently underestimated due to OTB and other substances present in coffee that interfere with the OTA antibodies. In this study, three LC methods with different extraction conditions and clean-up procedures were tested. Extraction in alkaline conditions led to conversion of OTA into open-ring OTA which is not recognised by OTA antibodies. On the other hand, OTB which was present in samples of ground coffee containing more than 0.8 µg/kg OTA, is recognised by antibodies with a higher affinity than that of OTA, contributing to saturation of IAC columns, which finally leads to OTA underestimation.

The development and validation of OTA-including multi-toxin analytical procedures deserved the attention of an increasing number of research groups. For example, Brera *et al.* (2011a) reported the single-laboratory validation of an HPLC-FLD method for aflatoxins and OTA in baby foods and paprika. Frenich *et al.* (2011) developed a QuEChERS-based extraction procedure and UHPLC-MS/MS for aflatoxins, OTA, citrinin, BEA and enniatins A, A_1 and B_1 in eggs. Recoveries ranged from 70% to 100%, except for OTA, AFG_1 and AFG_2. Relative standard deviations were always lower than 25% and LOQ ranged from 1 µg/kg for AFB_1, AFB_2 and AFG_1 to 10 µg/kg for enniatin A, citrinin and OTA. An HPLC-MS/MS method with photoionisation was presented by Capriotti *et al.* (2010) for aflatoxins, OTA, ZEA and DON in cereals.

Most of the papers reporting the development of multi-toxin methods were completed with related surveys. Following on from that there are some studies that in general describe in-house validated methods with acceptable recoveries ranging consistently from above 60% to 110%, good precision values in terms of RSD always lower than 20% and LOQs at levels from less than 1 µg/kg up to a few µg/kg depending on specific mycotoxins. Their application in surveys demonstrates their potential for routine analysis. Thus, Rubert *et al.* (2011c) reported the development of an HPLC-MS/MS method for aflatoxins and OTA in tiger nuts and their beverages in Spain; the same research group described a method based on matrix solid phase dispersion and HPLC-MS/MS for aflatoxins and OTA in cereals in Spain (Rubert *et al.,* 2010); Ibañez-Vea *et al.* (2011a) developed a UHPLC-FLD for aflatoxins, ZEA and OTA in barley in Spain; Rahmani *et al.* (2010) validated an HPLC-FLD method for aflatoxins, ZEA and OTA in cereals in Malaysia; Jin *et al.* (2010) described a UHPLC-MS/MS method for determination of OTA and DON, 3-acetyl-

DON, 15-acetyl-DON, NIV, fusarenon X, moniliformin, ZEA, zearalanone (ZAN) and OTB in grains in China using $^{13}C_{15}$-deoxynivalenol as internal standard; Beltrán *et al.* (2011) described a UHPLC-MS/MS method for aflatoxins, AFM_1 and OTA in baby food and milk in Spain; Soleimany *et al.* (2011) gave an account of an HPLC-PDA/FLD method with PHRED and post-column derivatisation for aflatoxins, OTA, ZEA, DON, fumonisins, T-2 and HT-2 in cereals in Malaysia. Sørensen *et al.* (2010) described a method based on mixed-mode reversed-phase anion-exchange chromatography in direct ion-exchange mode followed by HPLC-MS/MS for OTA, fumonisins and mycophenolic acid in meat products in Denmark. A method based on HPLC-MS/MS was developed and applied to aflatoxins, OTA, fumonisins, ZEA, sterigmatocystin, cyclopiazonic acid and trichothecenes in cereals in Colombia by Martos *et al.* (2010), and Ediage *et al.* (2011) validated an HPLC-MS/MS method applicable to the analysis of OTA and another 24 mycotoxins in cassava flour, peanut cake and maize samples from Benin. In this study, all the toxins were extracted in a single step with a mixture of methanol/ethyl acetate/water (70:20:10). The LOQs ranged from 0.3 µg/kg to 106 µg/kg. BEA, which is not among the most studied mycotoxins, was detected in the maize samples (range <LOQ to 25 µg/kg).

Finally, Koch *et al.* (2011) gave an account of two recently developed certified reference materials for the determination of OTA in roasted coffee and red wine, which may help to fill the gap in the availability of these valuable and highly recommended tools for supporting analytical quality control.

9. Patulin

Several methods have been developed to determine patulin in apple products, and recently more selective analytical methods have been published that are based mainly on LC-MS or GC-MS. Rosinska *et al.* (2009) described a rapid and simple method for the determination of patulin in apple products. The sample was extracted with ethyl acetate and then the extract was cleaned-up by extraction with a sodium carbonate solution. Patulin was determined by reversed-phase HPLC using a C_{18} column and a photodiode array detector (DAD) and UHPLC with electrospray-tandem mass spectrometry. The LOD for patulin was found to be 1 µg/kg for the HPLC-DAD method and 10 µg/kg for the UHPLC-MS/MS method. Cunha *et al.* (2009) have developed and validated a method for the determination of trace levels of patulin using GC-MS in apple products and quince jam. The method was based on extraction of patulin with ethyl acetate/hexane, alkalinisation and silylation with *N,O*-bis-trimethylsilyl trifluoroacetamide with 1% of trimethylchlorosilane. The accurate determination of patulin was achieved by employing commercial $^{13}C_{5-7}$ patulin as an internal standard. The LOD and LOQ of the method using real samples were 0.4 µg/kg and 1.6 µg/kg, respectively. The method was successfully applied to the determination of patulin in apple fruit and apple products including juice, cider and baby food, and also in quince fruit and quince jam. A method was developed (Yang *et al.*, 2009) to clean up and detect total patulin in apple, hawthorn and tomato products using HPLC-MS/MS. Chromatographic separation was performed on a C_{18} column and determination was achieved by HPLC-ESI-MS/MS. A simple and sensitive method for the determination of patulin in fruit juice and dried fruit samples was developed using a fully automated method consisting of an in-tube solid phase micro-extraction coupled with LC-MS operated in selected-ion monitoring mode with a $^{13}C_3$-patulin internal standard (Kataoka *et al.*, 2009). Samples could be analysed directly with only dilution after filtration. ESI conditions in the negative mode were optimised for MS detection of patulin. The method was sensitive (LOD of 24 ng/l) and gave recoveries upward of 92%. This method was applied successfully for the analysis of fruit juice and dried fruit samples without chromatographic interference with the patulin peak. Farhadi and Maleki (2011) developed a simple and sensitive method based on dispersive liquid-liquid micro-extraction in conjunction with HPLC-DAD for the quantitative analysis of patulin in apple juice and concentrate samples. The recovery values were in the range of 94-97% and the detection limit was 4.0 µg/l.

An HPLC-MS/MS method for determining patulin in apple- and pear-based foodstuffs was developed by Desmarchelier *et al.* (2011). The sample preparation is based on the QuEChERS procedure involving an initial extraction step with water and acetonitrile. The clean-up was performed using dispersive solid-phase extraction with a mixture of magnesium sulphate, primary secondary amine sorbent, and *n*-octadecylsiloxane sorbent added to the extract. Quantitation was performed by isotope dilution using ($^{13}C_7$)-patulin as internal standard. The LOD and LOQ were ≤0.5 and ≤10 µg/kg, respectively. Sargenti and Almeida (2010) developed a simple and rapid method for patulin analysis in apple juice without prior clean-up. This method combined sonication and liquid extraction techniques and was used for the determination of patulin in 37 commercial apple juices. The ultrasonic technique used a combination of two non-miscible solvents together (water in the apple juice and ethyl acetate), that permitted a rapid extraction of patulin from juice using a reduced amount of sample and extraction solvent in only one step. The extract was analysed by HPLC-UV. The LOD was 0.21 µg/l and the LOQ was 0.70 µg/l. A method was developed and validated in-house for the detection and quantification of patulin in apple juice concentrate using a charge-coupled device (CCD) camera on thin-layer chromatography plates (Welke *et al.*, 2009). The CCD camera is sufficiently sensitive to detect the change in spot fluorescence intensity caused by small differences in mycotoxin concentration under

homogenous illumination from a UV light source. The quantification and detection limits were 14.0 µg/l and 0.005 µg per spot, respectively. The method was applied to the analysis of 16 apple juice concentrate samples and patulin levels ranged from 15 to 46 µg/l. This study demonstrated the applicability of the TLC-CCD camera technique as a tool for monitoring patulin in apple juice.

10. Trichothecenes

In the past year there have been several review articles published relating to trichothecene mycotoxins. The review by Li *et al.* (2011c) focuses on the toxicity and metabolism of T-2 and analytical methods used for determining T-2. Several methods for the determination of T-2 based on traditional chromatographic, immunoassay, or mass spectroscopic techniques are described. Meneely *et al.* (2011) published a review describing trends in analytical methods for determination of some of the major trichothecene mycotoxins, namely DON and T-2 and HT-2. The paper focused on cereals and cereal products with a particular emphasis on screening and rapid methods. Cano-Sancho *et al.* (2010b) concentrated on the potential use of biomonitoring as a means to assess exposure to *Fusarium* toxins, suggesting it would be more accurate than traditional methods such as monitoring dietary intake.

There were fewer publications about the use of gas chromatography (GC) for trichothecene analysis. Ibanez-Vea *et al.* (2011b) reported the use of an in-house validated method for simultaneous determination of eight type A and type B trichothecenes in barley. The method was validated in terms of selectivity, linearity, precision and accuracy, limits of detection and quantification and recovery. The within and between-day precision and recovery were established by spiking at three contamination levels. The procedure included extraction of trichothecenes with acetonitrile/water (84:16), clean-up with Multisep® columns, derivatisation and GC-MS analysis. Recovery ranged from 92.0 to 101.9% (RSD<15%), except for NIV (63.1%), and the LODs and LOQs ranged from 0.31 to 3.87 µg/kg and from 10 to 20 µg/kg, respectively. Valle-Agarra *et al.* (2011) reported the application of a method using capillary GC with electron capture detection (GC-ECD) for determination of HT-2 and T-2 in paprika. The method that gave the best recoveries used acetonitrile/water (84:16) as extraction solvent, clean-up by solid-phase extraction followed by clean-up on IAC. The LODs for T-2 and HT-2 were 7 and 3 µg/kg, respectively, and the recovery for paprika spiked with 1000 µg/kg was 80.1% and 71.1% for T-2 and HT-2, respectively. Of the 32 samples tested, 3 were found to contain HT-2 (maximum concentration 199 µg/kg) and 2 contained T-2 (maximum 11 µg/kg). Bankole *et al.* (2010) reported a survey of trichothecenes in Nigerian maize using GC-MS. Out of the 32 samples analysed for trichothecenes, only one sample was contaminated with 15-monoacetoxyscirpenol at 4 µg/kg, and two samples were contaminated with T-2 tetraol at levels of 73 and 280 µg/kg. No other trichothecenes were detected in any of the samples. Thus, in spite of the limited number of samples investigated, it seems trichothecene toxins do not appear to be major contaminants of Nigerian maize. GC-ECD was used in a study of the effects of sampling and extraction on the quantification of DON by Hallier *et al.* (2011). Numanoglu *et al.* (2011) investigated single and multiple-stage extraction procedures for DON. The results obtained showed that a single-stage procedure underestimated the DON concentration in maize by a factor of up to 24% depending on the initial DON level. It was concluded that, in general, two extraction steps were acceptable for the extraction of approximately 90% of DON from maize, although three steps would be required to achieve this for highly contaminated samples. The effect of extraction time on the amount of DON extracted was also investigated. Single stage extraction was performed for different time periods from 5 to 30 minutes. The results showed that average recovery increased from 5 to 15 minutes, then decreased; at 30 minutes the average amount of DON found was 12% less than at 15 minutes. The authors suggest that increased extraction time leads to increased extraction of food components that may result in a back diffusion of DON to the matrix. When the results were analysed statistically it was shown that extraction time had no significant effect on the DON levels found (P>0.05). The authors do not report the number of replicates carried out, so while the average values appear different the error bars for measured DON for different time periods overlap.

HPLC with various different detectors continues to be widely used and reported in the literature, the most common being UV or UV-DAD (for DON) and fluorescence (for T-2 and HT-2). An LC-DAD method for the determination of the T-2 and HT-2 in cultures of *Fusarium langsethiae* in oat-based and other *in vitro* media was described by Medina *et al.* (2010). Results showed that extraction with methanol/water (80:20) for 90 min, drying with nitrogen and analysis by LC-DAD was the fastest method for detecting HT-2 and T-2 production by *F. langsethiae* strains grown on oat-based media. Trucksess *et al.* (2010) published a method for DON in some major wheat food products, including bread, breakfast cereals, pasta, pretzels, and crackers. Samples were extracted with water containing 5% polyethylene glycol and cleaned-up by IAC. The toxins were then subjected to HPLC separation and UV detection. Recoveries of DON spiked at levels from 0.5 to 1.5 mg/kg in the five processed foods were >70%. Standard deviation and RSD values ranged from 2.0 to 23.5% and from 2.0 to 23.2%, respectively, showing the method was acceptable.

HPLC with UV detection has been used for surveys in developing countries, including the first reports of natural occurrence of DON in maize from Iran (Karami-Osboo

et al., 2010), cereals in Lebanon (Antonios *et al.,* 2010) and durum wheat in Tunisia (Bensassi *et al.*, 2010). The determination of DON and NIV in traditional Chinese medicine was also reported for the first time (Yue *et al.*, 2010). Thammawong *et al.* (2010) used an in-house validated HPLC-UV method to determine DON and NIV in a study to establish their distribution during milling of Japanese wheat artificially infected with *Fusarium*. HPLC with fluorescence has been used for a survey of T-2 and HT-2 in soybean and soy meal from Argentina (Barros *et al., 2*011). Samples were cleaned up by IAC, extracts were derivatised and then analysed by HPLC-FLD. The method was validated in-house, mean recoveries for T-2 within the spiking range 125-500 µg/kg, were 90.9 and 81.3%, and 70.2 and 77.5% for HT-2 for soybean and soy meal, respectively. Limits of quantification were 75 µg/kg. Only 2 samples out of 64 analysed were found to contain low levels of type A trichothecenes, while confirmatory analyses of the contaminated samples were performed by HPLC-MS/MS. This study demonstrated low incidences and levels of T-2 and HT-2 in soybean harvested among the areas in the Cordoba Province.

An interesting development was the publication by Soleimany *et al.* (2011) describing a new method for the simultaneous quantification of 12 mycotoxins using reversed-phase HPLC with a photodiode array (PDA) and fluorescence detector, PHRED and post-column derivatisation. The mycotoxins included DON, and T-2 and HT-2. The LODs were 6.2 µg/kg for HT-2, 9.4 µg/kg for T-2, and 18.7 µg/kg for DON. The samples were cleaned-up using a multifunctional IAC. The method validation data, including analysis of certified reference materials to check accuracy, showed good method performance. The mycotoxins were measured in two chromatograms, and the trichothecenes were all detected in the PDA chromatogram. The method was applied to different cereal samples from the Malaysian market and was shown to be capable of detecting all 12 mycotoxins in a single run, without the use of MS as a detection system. This is of future benefit to those laboratories that do not have access to MS instruments.

The technique that has seen the largest number of publications is liquid chromatography with mass spectrometric detection. Valle-Algarra *et al.* (2011) used HPLC-MS/MS for DON determination in paprika. The optimised method consisted of extraction with acetonitrile/water (84:16) followed by SPE clean-up. DON was found in 4 out of 32 samples, the average value found was 125 µg/kg, the maximum level found was 269 µg/kg. This is one of very few reports concerning the determination of a single toxin using HPLC-MS/MS, as the majority of publications report detection of several toxins simultaneously. Capriotti *et al.* (2010) described the validation of a liquid chromatography/photoionisation MS/MS method for nine selected mycotoxins in wheat and maize samples. The analytes were chosen based on European Commission Regulation (EC) No. 1881/2006 and included DON, T-2 and HT-2. The authors reported moderate noticeable signal suppression for all analytes and internal standard. When this matrix effect was checked with an ANOVA test it did not give significant differences at P=0.005. Thereafter quantification was performed using calibrant standard solutions (not matrix matched).

Ediage *et al.* (2011) described an HPLC-MS/MS method for the simultaneous detection and quantification of 25 mycotoxins in cassava flour, peanut cake and maize samples. All toxins were extracted in a single step with a mixture of methanol/ethyl acetate/water (70:20:10). The LOQs varied from 0.3 µg/kg to 106 µg/kg. Guhsl *et al.* (2010) developed a multi-method for thirteen toxins including DON, 15-acetyl-DON, 3-acetyl-DON, T-2 and HT-2. Again a single extraction was employed, and no clean-up necessary. Matrix matched standards used as food matrices were shown to interfere with detection. Other authors report the use of different clean-up methods before HPLC-MS/MS analysis. Romagnoli *et al.* (2010) described a method for the simultaneous determination of DON, ZEA, T-2 and HT-2 in foodstuffs using a new multi-mycotoxin IAC available on the market (DZT MS-PREP®; R-Biopharm, DArmstadt, Germany). The authors claim the advantages of combining IACs and the HPLC-MS/MS technique include efficient removal of matrix interferences, clean chromatograms, high selectivity, low LODs and separation of a wide range of molecules with different physico-chemical properties in a single run. Schenzel *et al.* (2010) presented the first validated method for analysis of over 30 mycotoxins in drainage, river, and wastewater treatment plant effluent water. The method included solid-phase extraction over Oasis HLB cartridges, followed by LC-ESI-MS/MS. For the first time, BEA and NIV were quantified in drainage and river water samples with mean concentrations of 6.7 and 4.3 ng/l, and 6.1 and 5.9 ng/l, respectively.

Other developments reported include the use of UHPLC and hydrophilic interaction chromatography (HILIC). In addition there has been an increase in the number of publications reporting methods to determine the so-called 'masked' mycotoxins, such as DON-3-glucoside (DON-3-Glc). Jin *et al.* (2010) described an UHPLC-MS/MS method for simultaneous determination of 10 mycotoxins in grain. These were: DON, 3-acetyl-DON, 15-acetyl-DON, NIV, fusarenon X, moniliformin, ZEA, ZAN, OTA and OTB. Accurate determination was achieved by using commercial $^{13}C_{15}$-DON as internal standard, which compensated for target loss and eliminated matrix effects. Skrbic *et al.* (2011) reported an HPLC-MS/MS method to test Serbian grain samples for a range of *Fusarium* toxins, including DON-3-Glc. A simple one-step method was used to prepare the samples. Desmarchelier and Seefelder (2011) also reported an LC-ESI-MS/MS method to determine

DON and DON-3-Glc, while Vendl *et al.* (2010) reported the analysis of 84 cereal-based samples for DON, ZEA, DON-3-Glc, 3-acetyl-DON, ZEA-4-glucoside, α-zearalenol (α-ZOL), β-zearalenol (β-ZOL), α-zearalenol-4-glucoside, β-zearalenol-4-glucoside, and zearalenone-4-sulphate using HPLC-MS/MS. HILIC-HPLC-MS/MS was used by Beyer *et al.* (2010) to measure DON and DON-sulphonate, which is produced following treatment of contaminated feed with sodium bisulphite. Samples were analysed using stable isotope-labelled standards.

An interesting method for the detection of DON using the fluorescence excitation-emission matrix (EEM) was reported by Fujita *et al.* (2010). EEM is a graph composed of an excitation wavelength axis, an emission wavelength axis, and a fluorescence intensity axis. It is acquired by measuring the fluorescence intensity of a sample at consecutive excitation and emission wavelengths. The EEM of aqueous DON solutions at seven concentration levels showed fluorescent peaks, which were non-existent in water, particularly in the excitation (Ex) and emission (Em) wavelength ranges: Ex 200-240 nm/Em 300 nm and Ex 250-300 nm/Em 400-500 nm. Results showed that DON solutions could be distinguished from water, and the concentration of DON in the aqueous solution could be judged.

There was a smaller number of publications describing developments in immunological or biological based assays this year. Zhou *et al.* (2011) published a paper describing quantitative determination of DON using luminescent proximity homogeneous assay (AlphaLISA). This is an indirect competitive assay using anti-DON polyclonal antibody and coating antigen DON-BSA for detection of DON in cereals. Zhang *et al.* (2010) described the use of a colloidal-gold immunochromatography assay in mycotoxin detection. This article reviews the major mycotoxins contaminating cereal and oil foods and the application of colloidal gold immunochromatography assay in the detection of mycotoxins.

The synthesis of a T-2 MIP and its application in food analysis were reported for the first time. Molecularly imprinted solid-phase extraction (MISPE) procedures were optimised for further application in the analysis of T-2. The performance of the MIP throughout the clean-up of spiked maize, barley and oat sample extracts was compared with the results obtained when using non-imprinted polymer, OASIS HLB(R) and IAC. Although the OASIS HLB(R) SPE cartridge gave the highest recoveries, the precision was lower than the other methods. The MIP was superior in other aspects: selectivity towards T-2, matrix effect, multiple use and low limits of detection. A study on matrix effects was carried out by comparing the instrument response of a calibration solution with the same amount of T-2 added to blank extract. These ion signal recovery experiments showed that the most effective purification was IAC, although especially for barley and maize, the MISPE is a valuable alternative. The MIP has the significant advantage over all other clean-up methods of being able to be regenerated. The same MIP was used 200 times, and there was no visible loss of binding activity or remaining analyte after elution leading to false positives. Therefore the imprinted polymer cartridge has the potential to be much less costly than other clean-up methods. An analysis of 39 naturally contaminated samples (maize, barley and oat) by HPLC-MS/MS demonstrated that the MIP could be an excellent alternative for clean-up of contaminated food samples (De Smet *et al.*, 2010).

11. Zearalenone

While this section mainly comprises newly developed analytical methods for the determination of ZEA and its metabolites, three excellent reviews were recently published, covering various other aspects of the mycotoxin. The worldwide occurrence of ZEA and its exposure to humans has been summarised by Maragos (2010). Metzler *et al.* (2010) addressed the biological function of ZEA and its metabolites as endocrine disrupting chemicals. In particular, the metabolism of these compounds in fungi, plants and mammalian systems as well as their pharmacokinetics are described. Last but not least, *Fusarium* head blight disease of wheat – which results in ZEA contamination of food and feed – was discussed, especially toxigenic *Fusarium* species, their morphology and molecular identification methods (Stepien and Chelkowski, 2010).

For the determination of ZEA, HPLC-MS/MS based methods remain extremely popular. For instance, an UHPLC-MS/MS method for the quantification of ZEA, α-ZOL, β-ZOL, ZAN, α-zearalanol and β-zearalanol in traditional Chinese medicines was presented (Han *et al.*, 2011). After extraction with acetonitrile/water/sodium chloride, sample clean-up employed self-made SPE columns containing alumina base, florisil and kieselguhr. ESI was applied in negative mode and the analytes were measured on a triple quadrupole MS in selective reaction monitoring mode with [$^{13}C_{18}$]-ZEA as internal standard, which was added before sample extraction. The average recoveries of the mycotoxins, determined by standard addition, in rhizomes, roots, grasses, leaves, flowers and seeds were between 87 and 114%. Another method described the detection of ZEA, α-ZOL and β-ZOL in porcine follicular fluid using HPLC-ESI$^-$-triple quadrupole MS (Sambuu *et al.*, 2011). Samples were pre-treated with β-glucuronidase/arylsulphatase and purified with both C_{18}-SPE and IAC prior to analysis. While the quantification in the sub-µg/kg range seems doubtful, the sheer occurrence of ZEA- and α-ZOL-glucuronides in the follicular fluid of gilts on a natural diet is interesting.

The vast majority of recently described analytical methods for the determination of ZEA are HPLC-MS/MS based multi-mycotoxin methods. Desmarchelier *et al.* (2010) devised two different clean-up strategies – one using a QuEChERS approach and one using ASE – for the determination of 17 mycotoxins, including ZEA, in cereal-based commodities. A triple quadrupole MS was used in positive ESI mode for all mycotoxins, except for ZEA, for which the polarity was switched to negative during its retention time window. In nine validated matrices, the recovery of ZEA ranged from 49 to 113% with ASE, while the QuEChERS type clean-up allowed higher sample throughput and recovered 83 to 113% of ZEA. Lattanzio *et al.* (2011) determined nine mycotoxins by HPLC coupled with ESI triple quadrupole MS, using fully ^{13}C-isotope-labelled mycotoxins as internal standards in cereal-based foods. After extraction, samples were purified using a polymeric SPE column. ZEA recoveries ranged from 64 to 97% in barley, oat flour, durum wheat flour, rye- and wheat-based crisp bread. A UHPLC-MS/MS multi-method covering 26 mycotoxins was developed and validated for the analysis of tea and herbal infusions (Monbaliu *et al.*, 2010a). After extraction of the leaves with ethyl acetate/formic acid, a series of different SPE clean-ups followed prior to analysis using UHPLC-ESI$^+$-triple quadrupole MS. ZAN was used as internal standard, yielding an apparent recovery of ZEA from 91 to 98%. Only one out of 91 samples tested positive for a mycotoxin (FB_1). A UHPLC-MS/MS method for the determination of 10 mycotoxins, including ZEA and ZAN, in grains was presented by Jin *et al.* (2010). Samples were extracted with aqueous acetonitrile and purified by self-made cartridges, containing silica gel, florisil and kieselguhr. After reversed-phase separation employing a basic mobile phase, all analytes were ionised in the negative ESI mode. Detection was performed using a triple quadrupole MS in selective reaction monitoring (SRM) mode with [$^{13}C_{15}$]-DON as internal standard. The authors concluded from the higher recoveries obtained using internal calibrations that [$^{13}C_{15}$]-DON is a suitable internal standard for all measured mycotoxins, including ZEA. This approach is highly questionable, as different matrix effects clearly will lead to a falsified (not only uncorrected) result when retention times are not matching. Recovery of ZEA from spiked maize samples was 63-78%. Martos *et al.* (2010) presented a LC-ESI-MS/MS multi-method, measuring 22 mycotoxins in wheat, barley, oats, rye and maize. Rapid chromatography was carried out on a 7.5 mm mini-column, minimising the total run times to 11 min. Capriotti *et al.* (2010) developed and validated an HPLC-MS/MS method for the determination of nine mycotoxins in wheat and maize. Atmospheric pressure photoionisation was used in positive and negative mode (for OTA, DON and ZEA) to generate ions. Acetone (10%) was added to the mobile phases as a dopant to enhance ionisation. Despite a seemingly acceptable performance also for DON, the addition of 10% acetone to the mobile phase results in a very low capacity factor (k'<2) and therefore in possibly insufficient separation for early eluting compounds. Ediage *et al.* (2011) developed a method for the quantification of 25 mycotoxins in cassava flour, peanut cake and maize samples. A mixture of methanol/ethyl acetate/water was used to extract the analytes. After a defatting step, a clean-up on an aminopropyl SPE column was performed. An HPLC-ESI$^+$-triple quadrupole MS was employed in SRM mode, using ZAN as internal standard for ZEA (and other mycotoxins). Recovery of ZEA was 85-91%. Schenzel *et al.* (2010) presented a method to quantify some 30 mycotoxins – including ZEA, α-ZOL and β-ZOL – in drainage, river and wastewater treatment plant effluent water. After enrichment using SPE columns, mycotoxins were detected employing HPLC-ESI-triple quadrupole MS by two LC runs. D_6-ZEA was used as internal standard. Two LC-ESI-MS/MS methods were presented using multi-mycotoxin IACs for DON, T-2, HT-2 and ZEA to purify breakfast cereals and baby food (Romagnoli *et al.*, 2010) or wheat and biscuits (Tanaka *et al.*, 2010). The former method used polarity switching to deprotonise ZEA, while the latter analysed all mycotoxins in positive ion mode. Recoveries of ZEA ranged from 75-78% in breakfast cereals and baby food and from 88-109% in wheat and biscuit, for the two methods respectively.

Two high resolution MS methods for the determination of multiple mycotoxins were found in last year's literature. Zachariasova *et al.* (2010) developed a method for the control of 32 mycotoxins in beer. After protein precipitation with acetonitrile, beer samples were measured using UHPLC-ESI-Orbitrap-MS in both modes of polarity using two chromatographic runs. [$^{13}C_{18}$]-ZEA was used as internal standard. Recoveries of 89-117% were obtained for ZEA, 88-114% for α-ZOL and 89-111% for β-ZOL. Vaclavik *et al.* (2010) used DART ionisation coupled to Orbitrap-MS for the rapid quantification of mycotoxins in wheat and maize. A modified QuEChERS procedure was used to clean up the cereal samples and [$^{13}C_{18}$]-ZEA was used as internal standard for ZEA. As DART is not compatible with a previous chromatographic separation of analytes and matrix, cycle times of just about 20 seconds were obtained. Corrected recoveries of 93-115% were achieved for ZEA. While the sensitivity of the direct method is worse than for HPLC-MS/MS methods, 100 µg/kg ZEA could still be quantified.

While HPLC-FLD offers a limited range of analytes compared to HPLC-MS/MS, it can achieve comparable sensitivity and selectivity at lower instrument acquisition costs. Several publications described the use of HPLC-FLD for the determination of ZEA. Trucksess *et al.* (2011) developed and validated a method to quantify ZEA in botanical dietary supplements, soybeans, grains and various grain products after IAC clean-up. Recoveries of ZEA ranged from 81% to 88% for all tested commodities.

Rahmani *et al.* (2010) described an HPLC-FLD method for the simultaneous determination of ZEA, OTA and aflatoxins in cereals, after multifunctional IAC clean-up. Recoveries were 78-109% for all mycotoxins (82-105% for ZEA). Very similar to this work, an UHPLC-FLD method using the same clean-up to quantify ZEA, OTA and aflatoxins in barley was presented (Ibanez-Vea *et al.*, 2011a). Also the observed recoveries and the sensitivity of the method were almost identical. Soleimany *et al.* (2011) introduced an HPLC-UV-FLD method with a photochemical reactor for enhanced detection and post-column derivatisation for the simultaneous detection of 12 mycotoxins in cereals. A multifunctional IAC was used to clean up ZEA along with aflatoxins, OTA, DON, fumonisins, T-2 and HT-2. Recoveries from 72-111% were obtained from spiked maize samples. Belajova and Rauova (2010) validated several mycotoxin methods, including a HPLC-FLD method after IAC clean-up to determine ZEA in cereals and cereal-based foods. The recovery was 89-103% for ZEA. Lucci *et al.* (2010) used MIP-SPE for the clean-up of ZEA in cereal sample extracts, prior to HPLC-FLD measurements. The capacity of the MIP columns (regarding the amount of ZEA which can be applied to the columns before breakthrough) was estimated to exceed that of a common IAC column by a factor of four. Recoveries between 82 and 87% were obtained for wheat, while 86-90% were recovered from spiked maize samples.

Two more chromatographic methods were published. Wu *et al.* (2011) used HPLC coupled to evaporative light scattering detection to determine ZEA in barley after QuEChERS clean-up. Recoveries from 83% to 92% and a LOD of 1.6 µg/kg were obtained. In contrast to the often used HPLC methods, ZEA was also determined using GC-MS in aqueous environments (Dudziak, 2011). After SPE clean-up, samples were derivatised via silylation. Enrichment led to sub-ng/kg sensitivity with recoveries exceeding 62% in all observed cases.

Several novel immunoassays for the determination of ZEA were developed as well. Hervas *et al.* (2011) presented a magnetic bead-based electrochemical immunoassay on microfluidic chips to monitor infant foods. Sequentially, the immune-interaction (using competitive ELISA conditions) and enzymatic reaction with horseradish peroxidase (HRP) were performed on a microchip. Excellent recoveries of 101-103% were obtained, while the analysis time was just about 15 min. A very similar method was already proposed earlier (Panini *et al.*, 2010a). Again, antibodies immobilised on magnetic beads were used in a microfluidic system to bind ZEA in a competitive direct immunoassay. HRP was used as the linked enzyme to oxidise a substrate, the back reduction of which was electrochemically measured. The same group published another article presenting an immunosensor to determine ZEA in maize silage samples (Panini *et al.*, 2010b). This sensor is integrated in a continuous-flow system, where anti-ZEA antibodies are immobilised on a rotating disk. Again, HRP-linked ZEA was used to compete with ZEA and the enzymatic reaction was measured electrochemically within 15 min. Zhang *et al.* (2011c) developed a dual-label time-resolved fluorescence immunoassay for the simultaneous detection of ZEA and DON. Instead of fluorescent dyes, europium- and samarium-labelled antibodies were used. Both toxins were detected in the sub-µg/kg range, with recoveries of 83-108% for ZEA and 88-107% for DON. Peters *et al.* (2011) developed a multiplex flow cytometric microsphere immunoassay for ZEA, aflatoxins, OTA, DON, fumonisins and T-2. Colour-coded microsphere suspension array was combined with a specific flow cytometer. Mycotoxin-protein conjugates competed with free mycotoxins for specific antibodies. Strong matrix effects were encountered in feed samples, so a suitable multi-mycotoxin clean-up would strengthen the proposed method. Dorokhin *et al.* (2011) devised a prototype imaging surface plasmon resonance (iSPR) based multiplex competitive inhibition immunoassay for the simultaneous determination of ZEA and DON. Results of the iSPR assay were generated in less than 15 min and matched well with comparative HPLC-MS/MS measurements. Finally, Nabok *et al.* (2011) used total internal reflection ellipsometry to detect ZEA and T-2. Both direct and competitive immunoassays were tested, yielding detection limits in the sub-µg/kg range for these mycotoxins.

12. Mycotoxins in botanicals and spices

There were several surveys of mycotoxins in spices published this past year from Pakistan, Turkey, Korea and Malaysia. Aflatoxins in chilli peppers were surveyed from rural, semi-rural and urban environments from Pakistan (Iqbal *et al.*, 2010). The results showed that 23 (52.3%), 22 (50.0%) and 29 (65.9%) of samples from rural, semi-rural, and urban areas respectively, contained levels of aflatoxins which exceeded the European Union limits. The mean total aflatoxins in the chilli pepper samples were 27.7, 17.7 and 16.2 µg/kg, respectively from each region. The data indicated that individual localities have particular problems with mycotoxin contamination that is often greater than the limit set by the European Union. The aflatoxin contamination of ground red pepper in Turkey was surveyed from September 2008 to February 2009 (Set and Erkman, 2010). The results showed that 17.1% (14/82) and 23.1% (19/82) of unpacked ground red pepper were over the total aflatoxins and AFB_1 legal limits, respectively, while only one packed sample was over the legal limit of AFB_1 by 90 µg/kg. These results demonstrate the importance of analysing for aflatoxin in red pepper produced in Turkey. A survey of OTA in red paprika in Korea included 200 samples obtained from various commercial sources (Ahn *et al.*, 2010b). Approximately 30% of the red paprika samples were positive for OTA indicating a need to establish a

maximum level for regulation. OTA was determined in 120 commercial white and black pepper samples, in both powder and seed form, from Malaysia (Jalili *et al.*, 2010). The analysis method was based on HPLC-FLD with IAC clean-up. A total of 57 samples (47.5%) were contaminated with OTA ranging from 0.15 to 13.58 µg/kg. The results showed that there was a difference between types of pepper and brands, with the OTA in black pepper significantly higher than the white pepper. The highest level of OTA (13.58 µg/kg) was detected in a sample of black pepper seed, followed by 12.6 µg/kg in a sample of black pepper powder, both being bulk samples purchased from the open market in Malaysia.

All of the analytical methods for mycotoxins in botanicals published this past year used HPLC with a variety of different detectors. A method was described for the analysis of OTA and OTB in traditional Chinese medicines using UHPLC-MS/MS (Han *et al.*, 2010b). The matrix effects were minimised by using an [$^{13}C_{20}$]-OTA internal standard and a simple sample pretreatment. A total of 51 botanical samples widely used in China were analysed with this method for OTA and OTB with only 4 samples contaminated at low levels. It was concluded that these products presented a low risk of ochratoxin exposure to consumers who occasionally used traditional Chinese medicines. Another publication by the same authors described an UHPLC-MS/MS method for the simultaneous determination of AFB_1, AFB_2, AFG_1, AFG_2, AFM_1 and AFM_2 in traditional Chinese medicines (Han *et al.*, 2010c). The method utilises a [$^{13}C_{17}$]-AFB_1 internal standard and a solid phase purification process prior to the HPLC analysis. The observed recovery rates of the six aflatoxins ranged from 85.6% to 117.6% in different matrices. This method was used for the analysis of 30 commercial samples with 16 contaminated with aflatoxins with mean ranges of 0.50 to 1.40 µg/kg. Interestingly, aflatoxin M_1 was detected in 2 samples with the maximum level of 0.70 µg/kg. No samples were found positive for aflatoxin M_2. An HPLC method with UV detection was described for the simultaneous determination of DON and NIV in traditional Chinese medicine (Yue *et al.*, 2010). The authors evaluated different solid phase clean-up columns and extraction solvents to optimise the recoveries of both mycotoxins. The detection limits for DON and NIV were 62.5 and 50.0 µg/kg, respectively. The recoveries from different botanical samples spiked with DON and NIV at levels ranging from 0.5 to 10 mg/kg were 82.8-99.9% and 80.8-100.3%, respectively. None of the 30 commercially available traditional Chinese medicine samples analysed were found to contain any detectable amounts of these mycotoxins. T-2 and HT-2 were also determined in traditional Chinese medicines, using an HPLC method that employed evaporative light scattering detection (Tan *et al.*, 2011). For sample preparation, two solid-phase extraction clean-up procedures were developed using Florisil and MycoSep227. Limits of detection were 10 µg/kg. A total of 138 samples of 46 commercially available traditional medicines were analysed. Only one sample was found to contain T-2 at a level of 64.0 µg/kg; this was confirmed by LC-ESI-MS/MS. An HPLC-FLD method with IAC purification has been developed and validated for ZEA in 107 widely consumed Chinese medicinal herbs and related products collected from different regions of China (Zhang *et al.*, 2011d). Recoveries from 3 different medicinal herbs spiked with ZEA at levels ranging from 30 to 600 µg/kg were from 80.8 to 98.3%. Naturally occurring ZEA was only found in coix seed medicinal herb (all nine samples) with levels ranging from 18.7 to 211.4 µg/kg. This is the first report of ZEA contamination of a Chinese medicinal herb.

Two separate single-laboratory validations were published for the determination of mycotoxins in botanical matrices using HPLC-FLD with IAC purification. The accuracy, repeatability and reproducibility characteristics of a method for measuring levels of FB_1 (Oles *et al.*, 2010) and ZEA (Truckess *et al.*, 2011) in botanical root products were determined by an AOAC International single-laboratory validation procedure. For the FB_1 method validation, replicates of 10 test portions of each powdered root product (black cohosh, *Echinacea*, ginger, ginseng, valerian, *dong quai* and turmeric) at each spiking level (FB_1 at 0, 50, 100, 200 µg/kg) were analysed on 3 separate days. For the ZEA method validation, 10 replicates of black cohosh, ginger and ginseng at each spiking level (ZEA at 0, 50, 100, and 200 µg/kg) were analysed on 3 separate days. For both methods, the HorRat values were <1.3 for all matrices examined.

There were two analytical methods published for the determination of mycotoxins in spices utilising HPLC and GC. One publication was a single-laboratory validation study for the simultaneous determination of aflatoxins and OTA in paprika by HPLC-FLD (Brera *et al.*, 2011a). The RSD_r and recovery of this method were within values reported in the current EU regulations which confirmed the method was fit for purpose. The LOD and LOQ values were 0.002 and 0.200 µg/kg for AFB_1 and 0.012 and 0.660 µg/kg for OTA, respectively, in paprika. An optimised analytical method for the analysis of HT-2 and T-2 in paprika and chilli pepper using capillary GC with electron capture detection and another method for the determination of DON by LC-MS for the same matrices was published (Valle-Algarra *et al.*, 2011). The method for determination of HT-2 and T-2 that gave the best recoveries included extraction with acetonitrile/water (84:16), clean-up by a solid phase column followed by an IAC and derivatisation with pentafluoropropionic anhydride. The limits of detections for T-2 and HT-2 were 7 and 3 µg/kg, respectively, and the recovery rates for paprika spiked with 1000 µg/kg were 71.1% and 80.1% for HT-2 and T-2, respectively. For DON, the optimised method included an extraction with acetonitrile/water (84:16) followed by the use of a solid

phase clean-up column with HPLC-MS/MS detection. The LOD for this method was 14 µg/kg DON in paprika and the recovery rate was 86.8%.

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